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(54) Title: METHOD FOR INCREASING SECRETION OF PROTEINS IN EUKARYOTIC HOST CELLS

(57) Abstract

A method is provided whereby genetically altered cells exhibit increased levels of secreted protein, such as to provide increased yields of recombinant proteins. The cells are genetically altered to increase synthesis of ribosome receptors, which induces proliferation of components of the secretory pathway.

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METHOD FOR INCREASING SECRETION OF PROTEINS IN EUKARYOTIC HOST CELLS

FIELD OF THE INVENTION

The present invention generally relates to methods for increasing the secretory capacities of cells, and more particularly to the use of genetically altered eukaryotic cells having an increased expression of ribosome receptors so as to exhibit increased secretory (or intracellular protein transport) capacity.

The invention was made with government support under Grant No. GM-38538 awarded by the National Institutes of Health. The government has certain rights in this invention.

The subject application claims priority from U.S. Provisional Application Serial No. 60/051,721, filed July 3, 1997, titled "Induction of the Secretory Pathway in Cells."

BACKGROUND OF THE INVENTION

An objective of many biotechnology and pharmaceutical companies is the production of large quantities of recombinant proteins for use in therapeutic applications. Two examples receiving significant publicity in recent years are TPA (tissue plasminogen activator) and EPO (erythropoeitin).

Methods have been sought to increase the secretion of recombinant proteins from the cell culture systems being used to produce them.

For example, as noted in U.S. Patent 5,272,064, issued December 21, 1993, inventor Thomason, use of the more highly evolved eukaryotic host cell systems and yeast host cell systems for the recombinant production of platelet-derived growth factor B had typically resulted in the secretion of biologically active rPDGF B in relatively low levels and thus the inventor had turned to an *E. coli* expression system.

For several other examples, U.S. Patent 5,759,810, inventors Honjo et al., issued June 2, 1998, discloses a method for secreting a human growth hormone in an *E. coli* host cell by expressing a genetically engineered *E. coli* host

cell to produce enhanced amounts of glutathione reductase with the target recombinant protein. U.S. Patent 5,679,543, issued October 21, 1997, inventor Lawlis, describes fusion DNA sequences which when expressed in a filamentous fungus, are said to result in increased levels of secretion of the desired polypeptide, such as human tissue plasminogen activator, human growth hormone, human interferon, etc.

Although these aforementioned technologies may have been advantageous for secreting a number of proteins, several potential drawbacks may be inherent. The ability to secrete proteins from both prokaryotes and eukaryotes is dependent upon the presence of signal sequences. Although these can be added to non-secreted proteins or swapped between secreted proteins, evolution has likely selected the best signal sequence to match the mature protein being secreted. In other words, splicing a signal sequence onto a protein may enable its secretion, but may have little positive effect on the rate or amount secreted. More importantly, these technologies often do not address the ability of the host cells to perform a number of covalent modifications or processing steps often so important in producing biologically active recombinant proteins. To this end, the ability to develop host cells in which the totality of the secretory apparatus -- including all of the processing enzymes -- is upregulated would have the greatest advantage.

In many instances, the most economical cell culture systems (bacteria, yeast, insect cells, etc.) do not produce proteins that possess adequate biological activity. In these situations, use of mammalian cells, such as CHO (Chinese Hamster Ovary), are necessary. However, such mammalian cell systems are more expensive due to the limited number of cells that can be grown per liter of medium, and the expensive nature of the components of the growth medium itself.

Thus, a clear benefit to companies that must produce their proteins in cell cultures would be the ability of cultured mammalian cells to exhibit increased levels of secretion for the recombinant proteins being produced. But even with other types of eukaryotic cells, increased protein secretion is desirable. For

example, yeast has been an attractive alternative to recombinant protein production in cells other than *E. coli*.

SUMMARY OF THE INVENTION

We have been able to use expression of a ribosome receptor, and parts thereof, to induce membrane proliferation in various eukaryotic cells. The membranes that have been proliferated are those needed for protein secretion in the cell types. We have demonstrated that membrane induction results in an increased secretory capacity in these cells.

In one aspect of the present invention, a process for increasing the intracellular transport or secretion of a desired protein in a eukaryotic host cell comprises expressing a gene encoding the desired protein in a eukaryotic host cell that has been genetically altered. The genetic alteration is effective to express an increased amount of at least a portion of a ribosome receptor so as to increase either the intracellular transport (if the desired protein is a membrane-bound receptor or enzyme) or the secretion of the desired protein with respect to wild-type eukaryotic host cells.

Among applications contemplated for use of the inventive cells having the genetically engineered proliferation of ribosome receptors or fragments thereof (that is, of cells exhibiting increased secretory capacity) are for the production of proteins, such as proteins useful for therapeutic purposes. Yields of the desired proteins -- usually, but not always recombinant proteins -- can be increased through this technology. Thus, the invention provides a process to increase the secretory capacities of cells. In this way cells can be created with an increased ability to transport or secrete a recombinant protein of interest, or, cells already expressing a desired protein can be transformed by practice of the invention to reach previously unattainable levels of transport and secretion.

BRIEF DESCRIPTION OF THE DRAWINGS

In the drawings:

Figure 1A is the primary structure (amino acid sequence) of the canine ribosome receptor;

Figure 1B is a diagrammatic representation of constructs used in the studies outlined below;

Figure 1C illustrates the alignment of amino acid sequences deduced from cDNA clones encoding ribosome receptor homologues from murine and human sources (dashes indicate identities, letters in mouse and human indicate variance from the canine, dots indicate putative deletions);

Figure 2 is an electron micrograph of wild-type yeast, where prominently shown are three major organelles, the nucleus (N), mitochondria (M) and the vacuole (V);

Figure 3 is an electron micrograph of yeast showing rough membranes that are elaborated in response to the expression of the full-length ribosome receptor;

Figure 4 is an electron micrograph of yeast expressing a construct (ΔNT in Fig. 1B) in which the ribosome binding domain has been deleted from the receptor which resulted in a proliferation of smooth membranes;

Figure 5 is an electron micrograph of yeast expressing a construct that contains only the ribosome binding domain and the membrane anchor (ΔCT in Fig. 1B) that resulted in the most dramatic a proliferation of rough membranes;

Figure 6 is a Northern blot showing that stimulation of membrane proliferation induces the expression of genes encoding proteins specific to organelles along the secretory pathway, where the gene that has been transfected into each strain is indicated at the top of the figure, and each row represents the expression levels of a gene that encodes a protein known to participate in different organelle-specific aspects of the secretory process;

Figure 7 is an indirect immunofluorescence of control yeast (top row) as well as cells expressing the ΔCT construct (bottom row), and shown is staining with an anti-ribosome receptor antibody (left panels), an antibody against the resident ER protein Sec61p (middle panels), and an antibody against Gda1p, a resident protein of the Golgi complex (right panels);

Figure 8 is a growth assay demonstrating that cells expressing high levels of a gene encoding bovine pancreatic trypsin inhibitor (BPTI) are unable to grow unless transfected with genes encoding ΔCT or full-length ribosome receptor;

Figure 9 is an assay whereby levels of BPTI are measured in the culture medium of control yeast cells or of cells expressing ΔCT;

Figure 10 is an electron micrograph of control monkey COS-7 cells (Panel A, lower cell, and Panel C) and COS-7 cells transiently transfected with a plasmid encoding the full-length ribosome receptor (Panel A, upper cell, and Panel B);

Figure 11 shows immunofluorescent detection of the ribosome receptor in Chinese hamster ovary (CHO) cells that have been stably transfected with cDNA encoding the ribosome receptor;

Figure 12 shows immunofluorescent detection of the ribosome receptor in Chinese hamster ovary (CHO) cells that have been stably transfected with a cDNA construct that enables inducible expression of the ribosome receptor; and,

Figure 13 illustrates results of an assay for secreted alkaline phosphatase where cells described in Fig. 12 were transiently transfected with a plasmid encoding a secreted form of the enzyme alkaline phosphatase.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Broadly, one aspect of this invention is a process for increasing the intracellular transport or secretion of a desired protein in a eukaryotic host cell. By "intracellular transport" it is meant that the desired protein is conveyed by cellular processes toward the cell membrane. For example, among the many membrane-bound proteins that could be desirably increased in transport by practicing this invention are ones which carry out covalent modifications on proteins of commercial importance. Many processing steps are being carried out enzymatically by these proteins *in vitro*. Some illustrative, important enzymes whose production would be increased through a stimulated proliferation of the secretory pathway

would include ones involved in glycosylation (core and terminal), tyrosine sulfation, proteolytic cleavage, or the addition of lipid anchors.

Where the desired protein is a secreted protein, then practice of the inventive process increases secretion of that desired protein through the cell membrane. For example, among suitable candidates are recombinant proteins such as growth factors, clotting factors, lymphokines and other cytokines, hormones, protease inhibitors, and other serum proteins.

The subject invention can be carried out in substantially any eukaryotic host cell, whether derived from fungi, plant, protozoan, invertebrate, or vertebrate cells. Expression of genes encoding desired proteins in genetically altered eukaryotic host cells can be accomplished by genetically engineered constructions and methods now well known to practitioners in this field.

Yeasts have been employed in large scale fermentations for centuries, can often be grown to higher densities than bacteria, and are readily adaptable to continuous fermentation processing. Suitable cultured cell systems for production of recombinant proteins in yeast systems are well known, as described, for example, by U.S. Patent 5,618,676. On the other hand, there are often advantages to using vertebrate cells, particularly mammalian cells, although there are other instances where insect cells derived from Drosophila or moths are preferred and where viral vectors (baculovirus) can yield high levels of expression. Generation of chimeric proteins in baculovirus expression systems is well known. For example, U.S. Patent 5,770,192, issued June 23, 1998, inventors Cayley et al., describe recombinant baculoviruses which express a foreign protein that is secreted from insect cells infected with the recombinant baculovirus.

The most widely used mammalian cell line for protein production continues to be CHO (Chinese hamster ovary), although some companies rely on baby hamster kidney (BHK) lines for the production of certain proteins. For the secretion of recombinant antibodies, hybridomas (derived from fusions of plasma cell lines such as SP2/0 with antibody producing spleen cells) are most frequently used, as they are created by the antibody production process itself. Once the genes encoding a specific antibody can be cloned from hybridomas, it is then possible to

use stably-transfected CHO cells for further production. Recently "triomas" have been developed where hybridomas have been fused to human B cells as part of the "humanization" process of recombinant antibodies.

For mammalian cells, the promoter derived from the human cytomegalovirus (CMV) is most commonly used. High levels of expression can also be achieved using the respiratory syncytial virus (RSV) promoter, and although one of the first promoters to be used for expression of recombinant proteins, SV-40 is still broadly used. SV-40 promoters are common in systems using gene "amplification" technology. In this case constructs usually contain the dihydrofolate reductase (DHFR) gene, which allows selection for amplification using methotrexate.

In these various eukaryotic cell systems genetically altered cells with increased secretory capacities can be obtained by practice of the present invention. Such genetically altered eukaryotic host cells will be altered, or engineered, to express an increased amount of a ribosome receptor or a fragment thereof with respect to wild-type eukaryotic host cells. As a consequence, the totality of the secretory apparatus, including all of the processing enzymes, can be upregulated.

As previously reported by one of us, the deduced primary structure of the 180 kD canine ribosome receptor (RRp) indicated three distinct domains: an amino-terminal stretch of 28 uncharged amino acids representing the membrane anchor, a basic region (pI = 10.74) comprising the remainder of the amino-terminal half, and an acidic carboxyl-terminal half (pI = 4.99). The most striking feature of the amino acid sequence is a 10-amino acid consensus motif that is repeated 54 times in tandem without interruption in the amino-terminal positively charged region. Wanker et al., *J. Cell Biol.*, *130*, pp. 29-39 (1995), incorporated herein by reference.

The primary structure (amino acid sequence) of the RRp is shown by Fig. 1A. The nucleotide sequence for the RRp has been submitted to GenBank and is available as accession number X87224. With reference to Fig. 1B, RR stands for full-length ribosome receptor, MA stands for the membrane-anchoring region, RBD stands for ribosome binding domain, CT stands for the carboxyl-terminal

region. Shown as ΔCT is a truncation of the carboxyl-terminal domain, and shown as ΔNT is a construct harboring a deletion of the RBD. Further details, such as a restriction map, are described in Wanker et al. *supra*.

The primary structure of the ribosome receptor shown in Fig. 1A is deduced from the canine cDNA sequence. This protein appears very highly conserved in other species where sequence data is available. There is an ever-increasing number of snippets of cDNA, known as expressed sequence tags (EST), appearing in the databases. The most commonly posted mammalian sequences are from mouse and human sources. When translated into amino acids, a number of them align quite nicely with the sequence of the canine ribosome receptor. Due to its repetitive nature, it is difficult to align short ESTs within the N-terminal repeat-containing domain. However, as can be seen in Fig. 1C, a considerable number of human and mouse ESTs from non-repeat regions show a striking level of amino acid identity (>90%) over their entire length. For all of the ESTs shown in Fig. 1C, there are amino acid identities at 84% of the human and for 79% of the mouse sequences. At the very N-terminus of the protein, spanning the membrane-anchoring domain, amino acid identities between human, mouse, and canine sequences are in excess of 95%. The overall values for mouse are most likely even higher than 79% as the EST encoding amino acids 946 and 979 most likely has a sequencing error. Evidence from mouse genomic sequencing indicates that the mouse homologue has at least 45 of the 54 repeats found in the canine primary structure. In view of the very highly conserved nature of the ribosome receptor as between species, and since we have demonstrated aspects of the invention in mammalian cells with a canine ribosome receptor fragment, the species from which the ribosome receptor or fragment originates in practicing this invention is not believed to be of much consequence.

The above-noted 1995 publication characterized the ribosome receptor *in vivo* through expression in *Saccharomyces cerevisiae*; however, no recombinant proteins (other than the recombinant ribosome receptor itself) were expressed. It had previously been speculated that over-expression of RRp in yeast cells would lead to an increase in the number of ribosomes bound to the ER

membrane. With the subject invention, we demonstrate that eukaryotic cells that have been genetically altered, or engineered, to express an increased amount of at least a portion of the ribosome receptor will lead to an increased intracellular transport or secretion of a desired protein.

By practice of the invention the secretory capacity of eukaryotic cells (from yeast to human) can be increased through a stimulated membrane biogenetic event. A ribosome receptor, or portions of the ribosome receptor, is expressed in a stable fashion in the desired cell type. These cells take on the attributes of highly differentiated secretory tissues such as pancreas, liver or plasma cells. These engineered cells then become the host for the high level production of correctly processed secretory or membrane proteins of commercial importance. Operationally speaking, host cells may be transfected with a gene encoding the protein of interest whose expression is optimized to the limit of processing and secretory capacity of the host cell.

By practicing this invention, eukaryotic cells (from yeast to human) programmed with foreign DNA to produce a given protein for commercial use may be manipulated, by way of the invention's process, to achieve an increase in their ability to transport, process and potentially secrete such proteins. In operational terms, these cells may be transfected with a gene, or parts thereof, encoding a ribosome receptor. The cells will, as noted above, take on the attributes of secretory tissues and thereby result in increased transport, processing and secretion when compared to the unmanipulated original cell. Expression levels of the ribosome receptor constructs can be adjusted to be optimal for the transport, processing and secretion of the protein of interest.

As will be understood, a number of categories of "desired proteins" exist, whose intracellular transport and/or secretion could be enhanced through the use of the invention. Most obvious are recombinant proteins whose expression is based on the transcription of cloned cDNA driven by a suitable promoter. Based on current technology, such constructs are most often, but not always, integrated into the genome of the host cell for more stable and reproducible expression levels. The cDNAs encoding the recombinant proteins are often derived from sources other

than the host cell's complement of transcripts and would be defined as "heterologous" (e.g., a human cDNA expressed in CHO cells). Through the use of human host cells, homologous expression of human proteins could also be achieved. The transport, processing and secretion of artificial proteins, as exemplified by "humanized" monoclonal antibodies, can also be improved over existing technology through the use of the invention.

In practicing this invention, the expression of an increased amount of the desired protein may be inducible or non-inducible. Constitutive expression would typically be preferred in the routine commercial situation. However, as there must be an optimization between the synthesis of a recombinant protein and its ability to be transported, processed and secreted, placing either the recombinant protein, or the secretory apparatus under regulated control may help to facilitate such optimization.

Once the desired protein is obtained in increased amounts with respect to wild-type eukaryotic host cell, then it preferably will be purified either from the growth medium of genetically engineered host cells or by extraction and purification from the host cells directly. In the case of secreted proteins, known technologies for isolation from growth media can be utilized. In the case of intracellular proteins, or membrane bound enzymes, many biochemical purifications have been worked out.

Further, in practicing the invention, the recombinant DNA molecule introduced into the genetic material of the eukaryotic host cell can be incorporated into the genome of the eukaryotic host cell or not. For the moment, the ability to create cell lines that achieve stable expression of proteins over long periods of time, a necessary prerequisite for FDA approval, can only be achieved by integration into the host cell's chromosomes. Episomal (plasmid-based) transfection can lead to higher levels of expression, but using present technologies it is inherently unstable. Advances may come about in the future whereby episomal expression can be stabilized. In such cases, the increased levels of production of recombinant proteins would benefit from a host cell's having increased secretory capacity.

The invention will now be illustrated by the following examples and with reference to the Figures, which are intended to be illustrative and not limiting.

EXAMPLE 1

Preparation of Constructs and Expression of Proteins

To achieve proliferation of rough ER membranes (as in Fig. 5) as well as other elements of the secretory pathway in yeast (Figs. 6 and 7), the N-terminal half of the canine ribosome receptor (amino acids 1-826) was cloned into a pYEX-BX vector (Amrad Biotech) behind the promoter derived from the *CUP1* gene. The ribosome receptor was as described by Wanker et al., *Journal of Cell Biology, Vol. 130*, pp. 29-39 (1995), in which a cDNA encoding the 180-kD canine ribosome receptor was reported to have been cloned and sequenced with the sequence data being available from GenBank/EMBL/DDBJ accession no. X87224. 5×10^7 cells are transfected with 5 μg of DNA using the published LiAc/DMSO procedure. Expression of the gene was then controlled by the presence or addition of copper (Cu_2SO_4, 0.5mM) in the growth medium (SD).

Various other promoters can also be used in preparing constructs useful in practicing the invention. For example, instead of the *CUP1* gene, *ADH* is another gene whose promoter could enable a high level of gene expression and represents a suitable equivalent. It may also be possible to induce equivalent, or even higher, levels of membrane proliferation by manipulating the constructs to contain only the ribosome binding (repeat-containing) domain and a membrane anchor. Alternatively, constructs with fewer or more repeats may enhance membrane production, and thus secretory activity.

EXAMPLE 2

To demonstrate the invention in mammalian cells, full-length ribosome receptor cDNAs as well as the N-terminal half of the receptor (amino acids 1-826) were cloned into a pcDNA 3.1/Zeo vector (Invitrogen, San Diego,

CA), where constitutive gene expression is controlled by the CMV promoter. Transient transfections of mammalian cells (1.6 x 10^6 cells with 10 g of DNA), were carried out through electroporation, prior to growth on DMEM (+10% FBS) in 10 cm dishes.

CHO cells were transfected by electroporation with ScaI linearized expression constructs to enable integration into the host cell genome. Cell lines stably expressing proteins cloned into the pcDNA 3.1 vector were selected through the inclusion of the antibiotic zeocin (250 μg/ml) in the growth medium. Other animal cells successfully responding to transfection by increasing their endoplasmic reticulums (ER) complement include HeLa (human cervical carcinoma), COS-7 (African green monkey kidney) and 293 (human embryonal kidney) cells.

Constitutive expression constructs: A ΔCT cDNA fragment was ligated into the pcDNA 3.1/Zeo (-) expression vector by standard techniques.

Inducible expression constructs: A ΔCT cDNA fragment was ligated into the pIND expression vector (Invitrogen) by standard techniques. In the resultant constructs, transcription was directed from the ecdysone/glucocorticoid response elements and the minimal heat shock promoter.

Secreted protein construct: Expression vectors encoding Secretory Alkaline Phosphatase (pSEAP2-Control) were obtained from Clonetech (Palo Alto, CA). These constructs were introduced into the appropriate cell type using lipofection techniques (Fugene6, Boehringer, Mannheim; Lipofectamine Plus, Life Technologies, Gaithersburg, MD).

Cell culture conditions: CHO/K1 cells were grown in Ham's/F12 medium with 10% FBS. CHO-Ecr (ecdysone receptor-expressing) cells were grown in the same way as CHO/K1 but zeocin was added to a concentration of 250 μg/ml. 48 hours prior to transfection, cells were plated at 25% confluency, at 16 hours prior to transfection fresh medium was added, and in the case of CHO-Ecr, zeocin was omitted.

Transfections: 10 μg of linearized expression construct was introduced by electroporation into CHO/K1 or CHO-Ecr cells (Invitrogen). Cells (1.6 x 10^6) were transfected in a final volume of 400 μl of culture medium. After

the pulse, cells were transferred to 100 mm plates with 10 ml of culture medium. Following a 24-hour recovery time, medium was changed and selection applied by the addition of either 300 μg of zeocin (non-inducible constructs) or 250 μg/ml zeocin and 500 μg/ml G418 (inducible constructs). Cells were then allowed to grow for another 24 hours prior to clone selection.

Clone selection: Clone selection was carried out using high dilution and seeding in 96-well plates or by cylinder cloning discrete foci on 150 mm plates. Cells were initially screened for overexpression of p180-related proteins by immunofluorescence using anti-p180 antibodies. After the initial screen, positive clones were expanded for further characterization.

Assessment of transfection and upregulation of the secretory pathway was carried out morphologically as well as biochemically. The results of these studies are depicted in Figs. 10-13 and described herein.

In wild type yeast cells, (Fig. 2) there is an overall lack of observable membranous structures in the cytoplasm. By comparing this electron micrograph to ones shown as Figs. 3 and 5, dramatic changes are obvious in the cytoplasm. Large quantities of ribosome-studded membranes, reminiscent of rough endoplasmic reticulum and typical of actively secreting tissues, have appeared. In Fig. 4, where the ribosome binding domain has been deleted from the receptor that is being expressed, large quantities of membranes are still produced, as in Fig. 3, however there are no densely-stained particles (ribosomes) associated with the membranes. In Fig. 5, where yeast have been transfected with a construct encoding little more than the repeat domain and the membrane anchor, the greatest proliferation of rough membranes has occurred, leaving little if any unoccupied space in the cytoplasm.

It appears that the membranes induced and seen clearly in Figs. 3 and 5 represent rough ER. Further evidence supporting the notion that these membranes represent bona fide rough ER is shown in Fig. 6. The expression of

constructs that induce rough membranes induces the co-expression of genes encoding rough ER proteins. Moreover, the expression of genes encoding marker proteins further along the secretory pathway occurs in the case of membrane induction by the ΔCT construct. The uniqueness of the ability of ΔCT to selectively induce these genes is demonstrated by the relatively low ability of other constructs -- some of which induce smooth membrane proliferation in yeast (e.g., ΔNT) -- to enable similar changes.

Fig. 7 documents the fact that the proteins whose genes are expressed at higher levels in induced cells (Fig. 6) are also synthesized and incorporated into the correct organelle is demonstrated in Fig. 7. Indirect immunofluorescence using the appropriate antibodies show an upregulation in the appearance of ER, complete with one of its resident proteins (Sec61p), as well as an increase in the number of Golgi-like structures in the transfected cells containing the resident guanosine diphosphatase (Gda1) protein. Taken together, the data presented in Figs. 6 and 7 indicate that genes of the secretory pathway are expressed and incorporated correctly into organelles during membrane proliferation induced by the expression of (part of) the ribosome receptor.

Fig. 8 is a growth assay demonstrating that cells expressing high levels of a gene encoding the secretory protein bovine pancreatic trypsin inhibitor (BPTI) are unable to grow unless transfected with genes encoding ΔCT or full-length ribosome receptor. BPTI is expressed in these cells in a regulated manner; the use of a *GAL* promoter enables expression when cells are grown in galactose-containing medium, but not on glucose.

Fig. 9 is an assay whereby levels of BPTI are measured in the culture medium of control yeast cells or of cells expressing ΔCT. BPTI was expressed under *GAL* control as described in the legend to Fig. 9. Quantification of BPTI was carried out as described in the text below.

Most important to the utility of the inventive process is the correlation between the apparent upregulation of the secretory pathway and an increase in secretory capacity. As mentioned, when cells were transfected with a vector encoding bovine pancreatic trypsin inhibitor (BPTI) under the control of the

inducible GAL promoter, growth stopped when BPTI production was induced in wild-type cells (vector only) (Fig. 8). It is likely that this block was the result of blocking the secretory pathway with excess BPTI. The block was relieved in the case of cells where membrane proliferation (and hence the secretory pathway) was induced through expression of RBD containing constructs (full-length RRp and ΔCT). Membrane proliferation alone, as occurs in the case of the expression of ΔNT, was incapable of rescuing these cells from the BPTI block.

That the BPTI block was relieved by its secretion from the cells that had proliferated the secretory pathway is demonstrated in Fig. 9. In this case, a sensitive assay for BPTI's ability to inhibit the proteolytic cleavage of a substrate by trypsin indicated that a 400% increase in BPTI secretion had taken place in ΔCT-containing cells, compared to vector-only controls. This value may represent a minimum value in the cell's ability to upregulate its secretory capacity as neither the levels of BPTI, nor of ΔCT have been optimized as yet.

That which was observed in yeast can also occur in mammalian cells. The proliferation of rough membranes in the cytoplasm of transfected African monkey kidney (COS-7) cells is dramatically demonstrated in the electron micrographs displayed in Fig. 10. Panel A shows two cells at low magnification, one transfected (top) and one untransfected (bottom). Higher magnification of the transfected cell (panel B) shows extensive rough ER proliferation compared to the untransfected cell (panel C). In this case, the full-length ribosome receptor was used, however immunofluorescence data show that similar membrane proliferation occurs using the ΔCT construct (not shown). Double label immunofluorescence studies verified that expression of the ribosome receptor in these cells results in the proliferation of rough ER membranes that contain increased levels of other resident proteins whose synthesis appears induced in response to the transfection (not shown).

As many commercial applications rely on the use of Chinese hamster ovary cells (CHO) for secretion of important proteins, we established two CHO cell lines that were stably transfected with constructs encoding ΔCT as the inducer of membrane proliferation. In the first case, a vector was used that places ΔCT under

control of the constitutive human cytomegalovirus (CMV) promoter, whereas the second construct placed ΔCT behind an inducible hybrid promoter that is stimulated through including the insect hormone ecdysone or ecdysone-analogs in the growth medium.

Fig. 11 shows immunofluorescent detection of the ribosome receptor in Chinese hamster ovary (CHO) cells that have been stably transfected with cDNA encoding the ribosome receptor. Note extensive membrane proliferation in transfected cells. Left panel: Cells transfected with cDNA encoding the ribosome receptor. Right panel: Cells transfected with vector alone.

Fig. 12 shows immunofluorescent detection of the ribosome receptor in Chinese hamster ovary (CHO) cells that have been stably transfected with a cDNA construct that enables inducible expression of the ribosome receptor. Left panel: Cells that have not been induced to express the ribosome receptor. Right panel: Cells grown for 24 hours in the presence of inducer.

Fig. 13 illustrates results of an assay for secreted alkaline phosphatase where cells described in Fig. 12 were transiently transfected with a plasmid encoding a secreted form of the enzyme alkaline phosphatase. Quantities of the enzyme appearing the culture medium at 24 hours after induction were measured colorimetrically for control cells and for ones whose expression of the ribosome receptor had been induced.

Figs. 11 and 12 document the proliferation of the endoplasmic reticulum in cells that express ΔCT. In the case of cells whose membrane proliferation was driven by constitutive expression of ΔCT driven by the CMV promoter, an abundant rough ER staining pattern is observed in comparison to vector-only controls (Fig. 11). A similar picture was obtained in the case of cells stably transfected with ΔCT under control of the inducible promoter. In this instance, striking upregulation of rough ER was observed after 24 hours of growth in the presence of the inducer when compared to uninduced control cells (Fig. 12).

As was the case for yeast, we demonstrated that mammalian cells manipulated in the manner of the invention have an increased ability to secrete recombinant proteins. Thus, a secretory form of the enzyme alkaline phosphatase,

under the control of the constitutive SV-40 promoter, was transfected into the inducible CHO cell line described above. Experiments summarized in Fig. 13 indicate a two- to three-fold increase in secretion in cells upon induction of the gene encoding ΔCT. This is believed to be a minimum value for the increase in secretion, with greater levels achievable when the levels of alkaline phosphatase and the levels of ΔCT expression have been optimized.

It is to be understood that while the invention has been described above in conjunction with preferred specific embodiments, the description, examples, and figures are intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims.

It is Claimed:

1. A process for increasing the intracellular transport or secretion of a desired protein in a eukaryotic host cell, comprising:

expressing a gene encoding said desired protein in a genetically altered eukaryotic host cell, the genetic alteration being effective to express an increased amount of a ribosome receptor or a fragment thereof with respect to wild-type eukaryotic host cell, wherein intracellular transport or secretion of the desired protein is increased with respect to wild-type eukaryotic host cell.

2. The process as in claim 1 wherein the desired protein is a membrane-bound protein.

3. The process as in claim 1 wherein the desired protein is a secreted protein.

4. The process as in claim 1 wherein the genetically altered eukaryotic host cell is derived from fungi, plant, protozoan, invertebrate, or vertebrate cells.

5. The process as in claim 1 wherein the genetically altered eukaryotic host cell is derived from a mammalian cell.

6. The process as in claim 5 wherein the genetically altered eukaryotic host cell is derived from a human cell.

7. The process as in claim 1 wherein the genetically altered eukaryotic host cell is derived from a cultured CHO cell.

8. The process as in claim 1 wherein the desired protein is a recombinant protein.

9. The process as in claim 1 wherein the genetic alteration includes stably transfecting the wild-type host cell with a DNA molecule encoding at least a portion of a ribosome receptor.

10. The process as in claim 9 wherein the expression of increased amount of desired protein is inducible.

11. The process as in claim 9 wherein the DNA molecule includes coding for a plurality of ribosome receptor repeating units.

12. A method for enhancing production of a recombinant protein, comprising:

isolating a recombinant DNA molecule having a genetic sequence coding for at least a portion of a ribosome receptor; and,

introducing the recombinant DNA molecule into the genetic material of a eukaryotic host cell to form a genetically engineered cell, wherein the genetically engineered cell becomes capable of secreting increased amounts of a recombinant protein when the coding sequence therefor is expressed.

13. The method as in claim 12 further comprising maintaining the genetically engineered cell under cell culture conditions to secrete recombinant protein therefrom.

14. The method as in claim 13 further comprising purifying recombinant protein secreted from the genetically engineered cell.

15. The method as in claim 12 wherein the eukaryotic host cell is mammalian.

16. The method as in claim 12 wherein the introducing step includes stably transfecting the eukaryotic host cell.

17. The method as in claim 12 wherein the recombinant DNA molecule is incorporated into the genome of the eukaryotic host cell.

Amino Acid Sequence of RRp

```
    MDIYDT QTLGVMVFGG FMVVSAIGIF LVSTFSMKET SYEEALANQR   46
KEMAKTHHQK VEKKKKEKTV EKKGKTKKKE EKPNGKIPDH EPAPNVTILL   96
KDPVRAPAVP VAPTPVQPPV VIAPVATVPA MPQEKLAPSP KDKKKKEKKV  146
AKVEPAVSSV VNSVQVLASK AAILETAPKE VPMVVVPPVG AKAGTPATST  196
AQGKKAEGAQ NQSRKAEGAP NQGKKAEGAL NQGKKAEGAQ NQGKKVEVAP  246
NQGKKAEGGQ NQGKKVEGAQ NQGKKAEGTP NQGKKAEGAP NQGKKTDGAP  296
NQGKKSEGAP NQGKKAEGAQ NQGKKVEVAP NQGKKAEGGQ NQGKKVEGAQ  346
NQGKKAEGTP NQGKKAEGAP NQGKKTDGAP NQGKKSEGAP NQGKKVEGAQ  396
NQGKKVEGVQ NQGKKAEGAQ NQGKKAEGTS SQGRKEEGTP NLGKKAEGSP  446
NQGKKVEVVQ NQSKKVEGAP NQGKKAEGSQ NQGKKTEGAS NQGKKVDGAQ  496
NQGKKAEGAP NQGKKVEGAQ NQGKKAEGTP NQGKKAEGAQ NQGKKAEGAP  546
NQGKKAEGAP NQGKKAEGAP NQGKKAEGAP NQGKKAEAAP NQGKKAEGAP  596
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NQGKKGEGTP NQGKKSEGSP NQGKKVDASA NQSKRAESAP IQGKNADMVQ  746
SQEAPKQEAP AKKKSGSKKK GEPGPPDSDS PLYLPYKTLV STVGSMVFNE  796
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Figure 1A

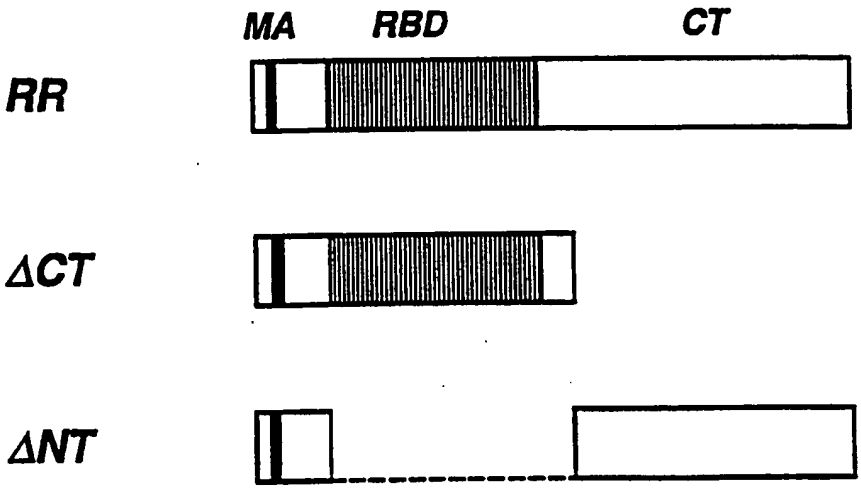
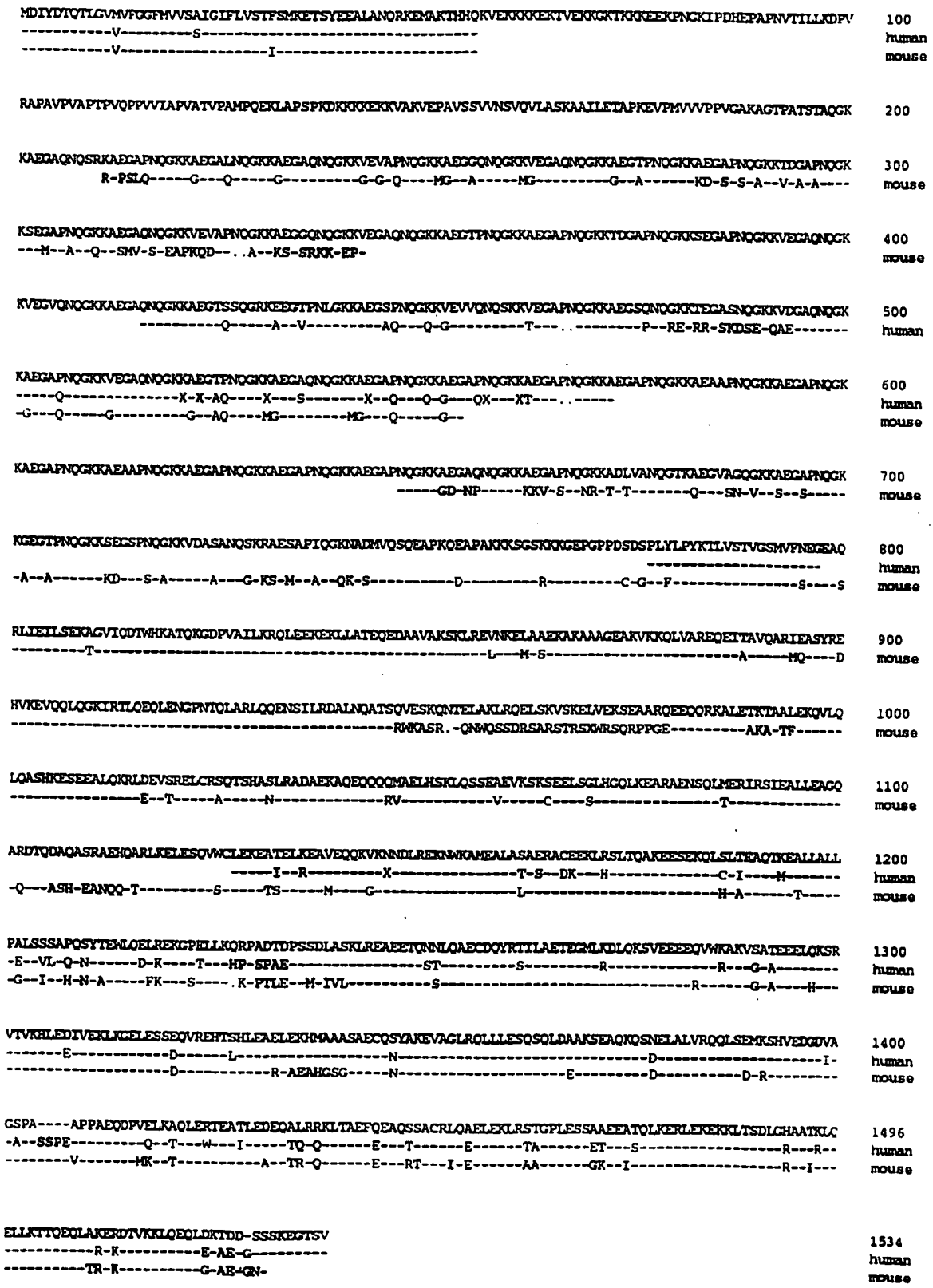


Figure 1B

Figure 1C



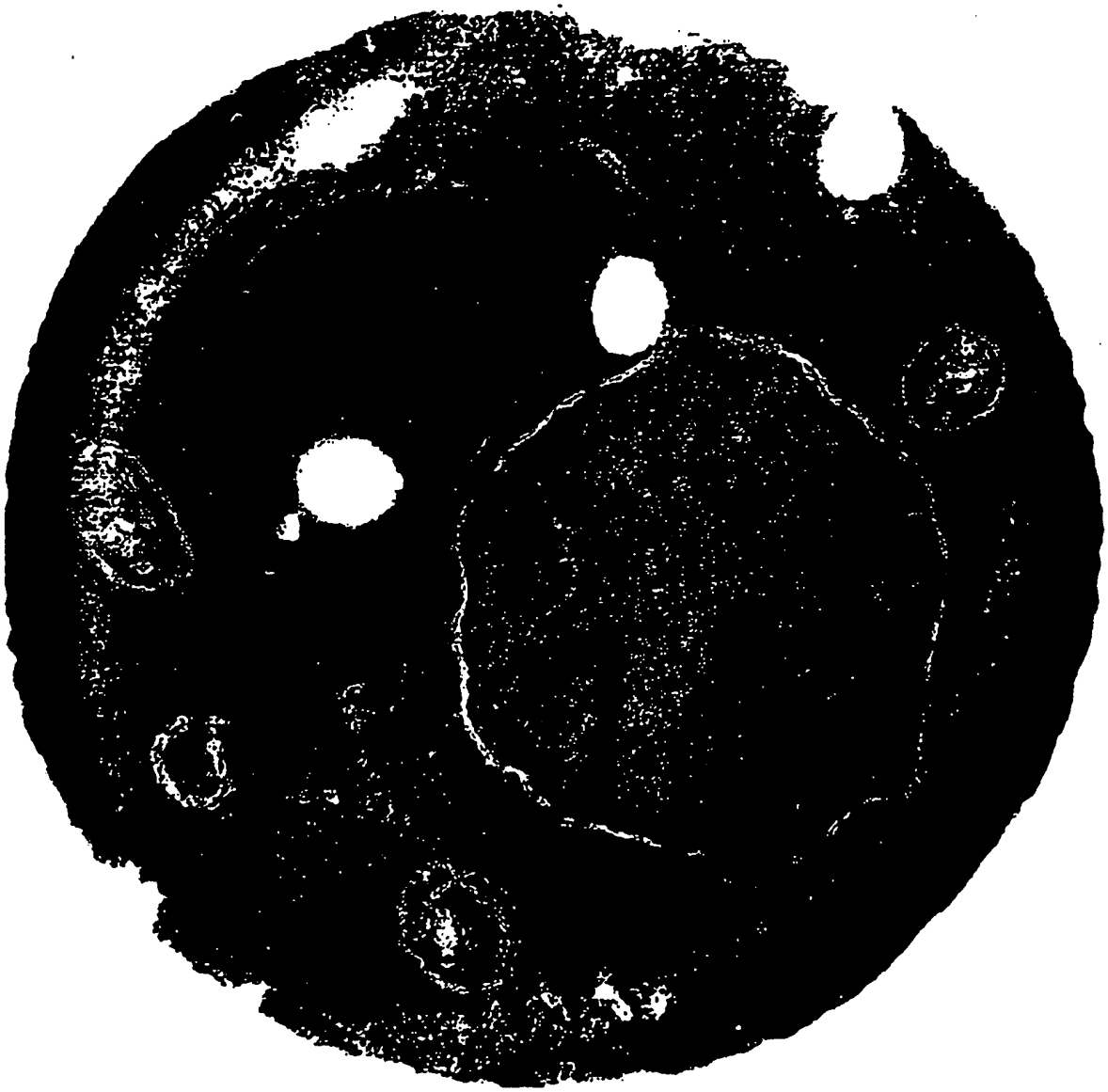
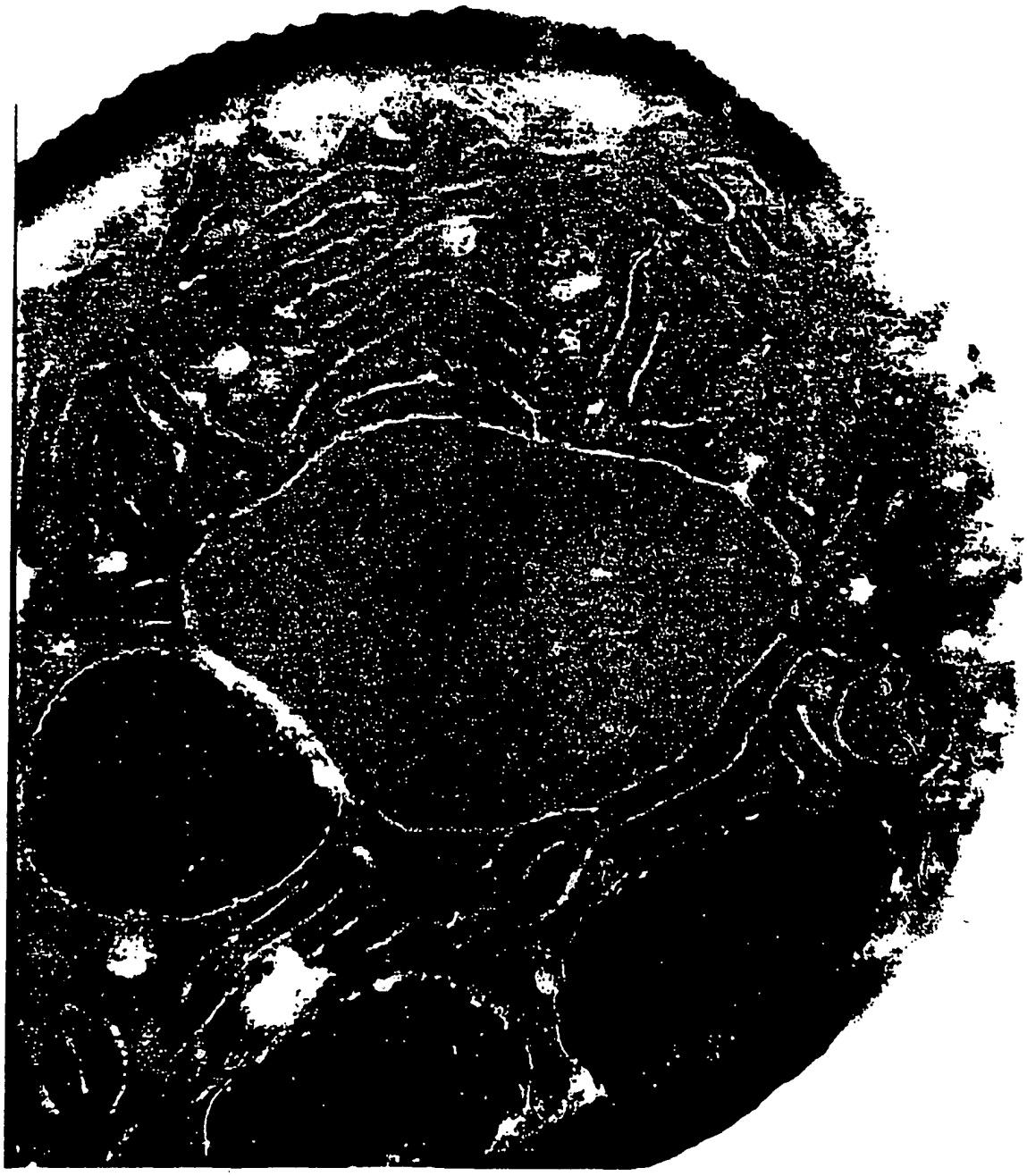


Figure 2



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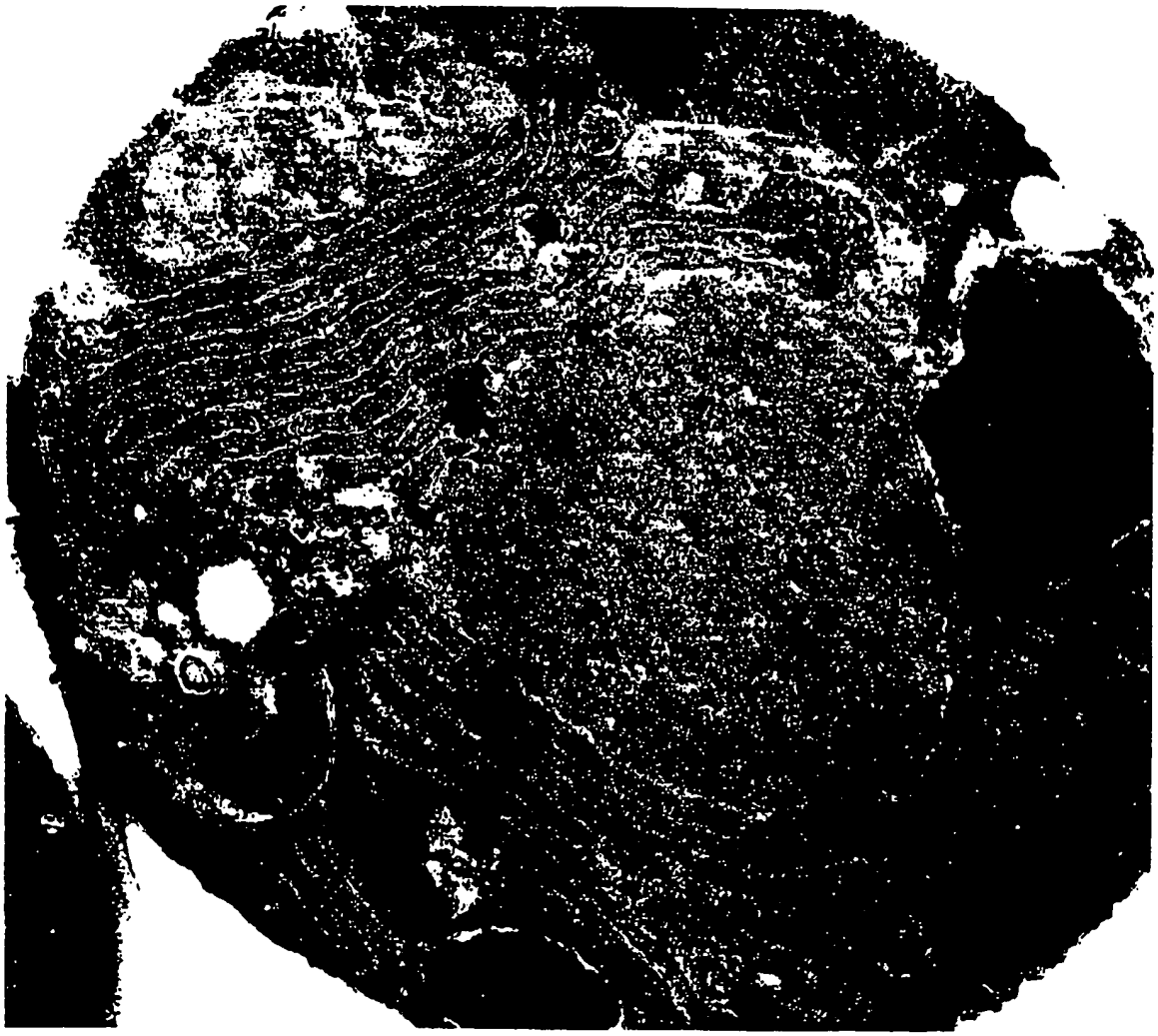

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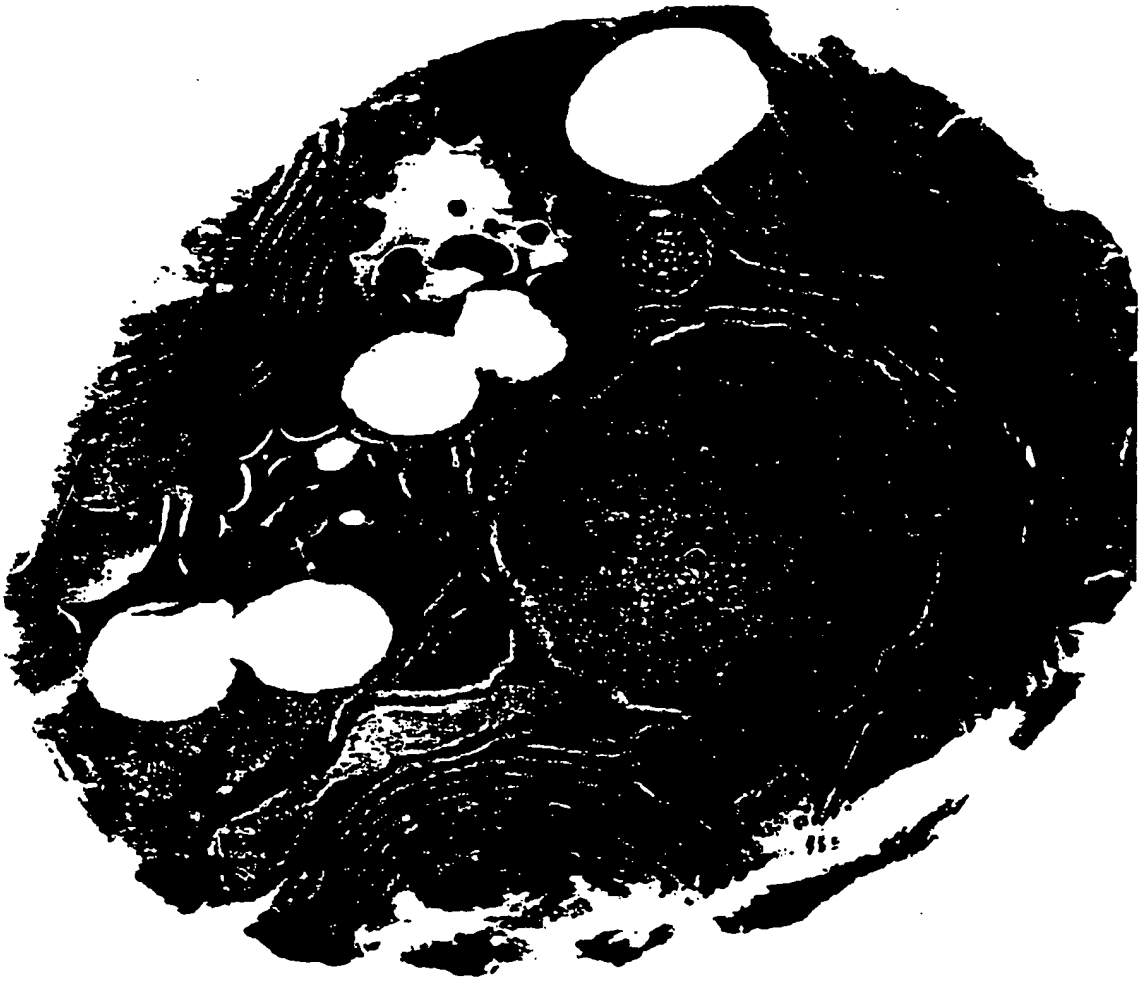

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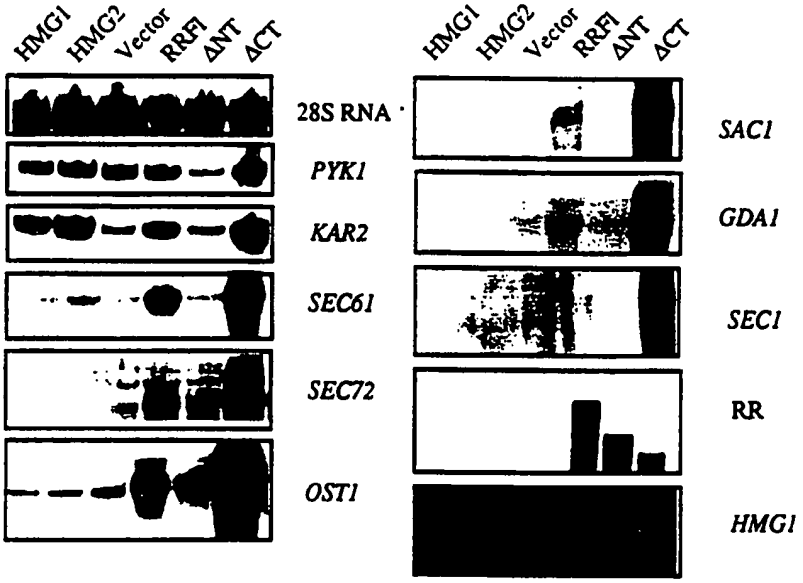
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ΔNT
ΔCT
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KAR2
SEC61
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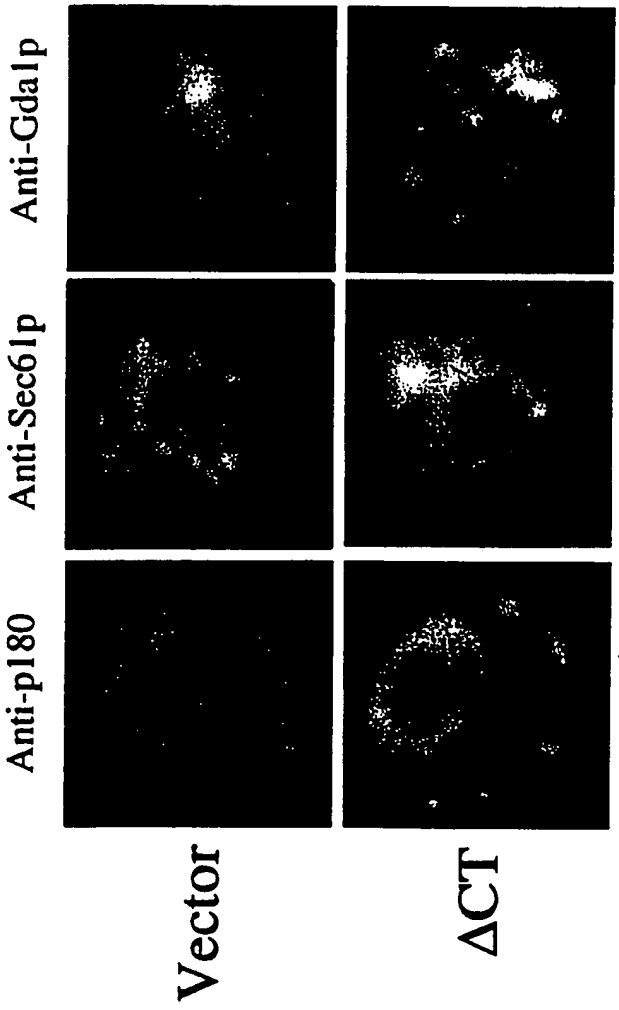
ΔNT
ΔCT
SAC1
GDA1
SEC1
RR
HMG1

Figure 6



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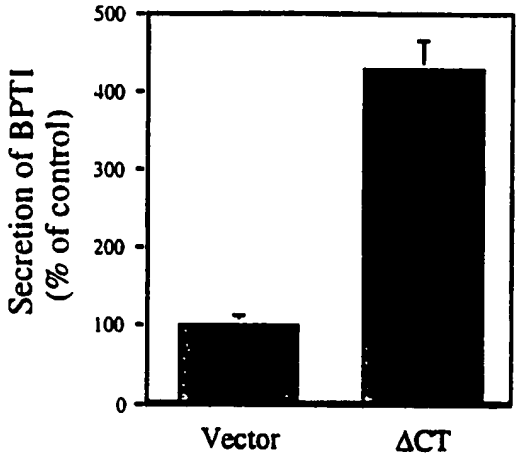
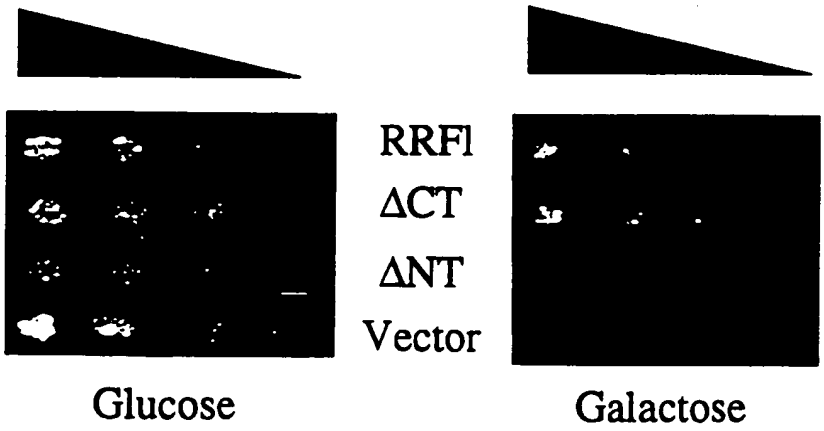


Figure 9



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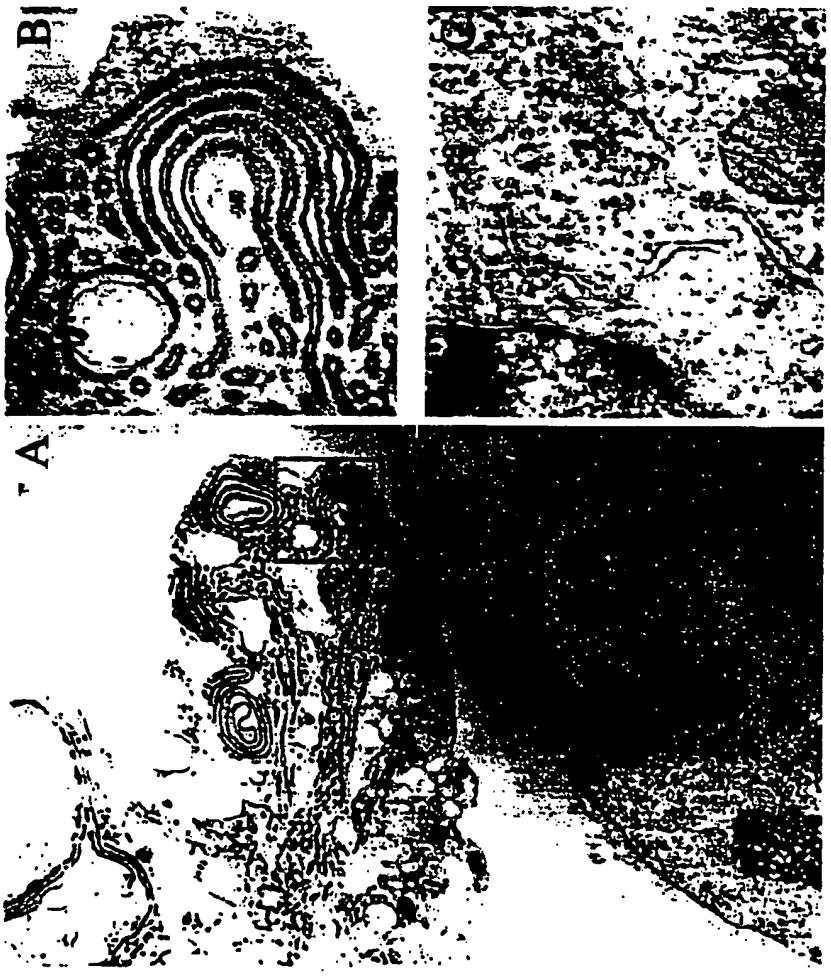


Figure 10

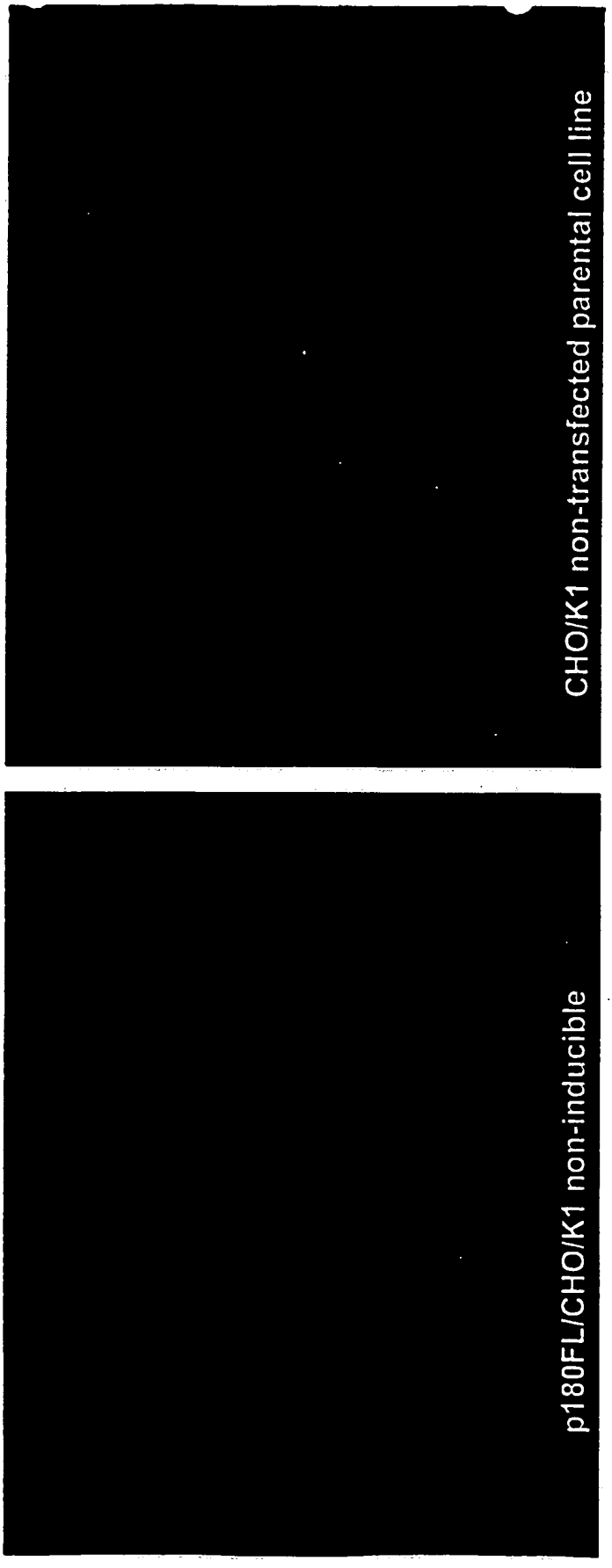
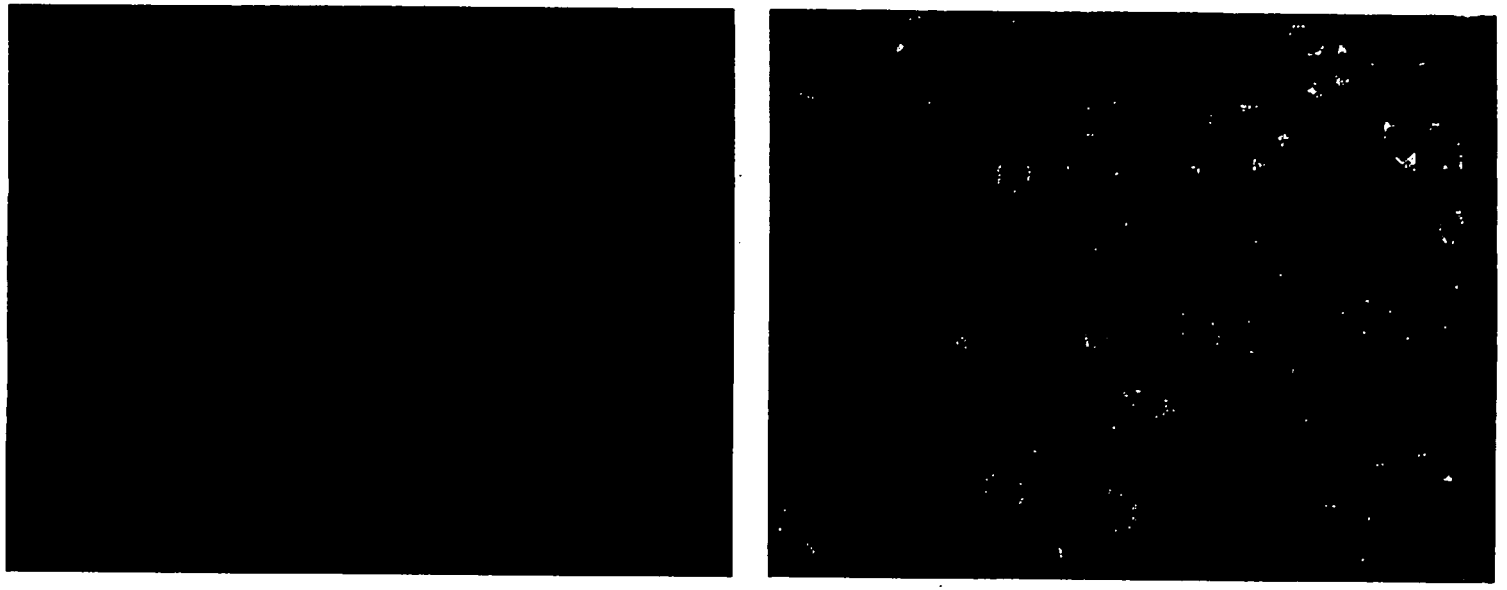

CHO/K1 non-transfected parental cell line
p180FL/CHO/K1 non-inducible

Figure 11

Inducible expression of p180 in stable transfected CHO cells



Uninduced

Induced
50μM Muristerone A, 24 h

Figure 12

Increase of the Secretory Capacity in CHO cells

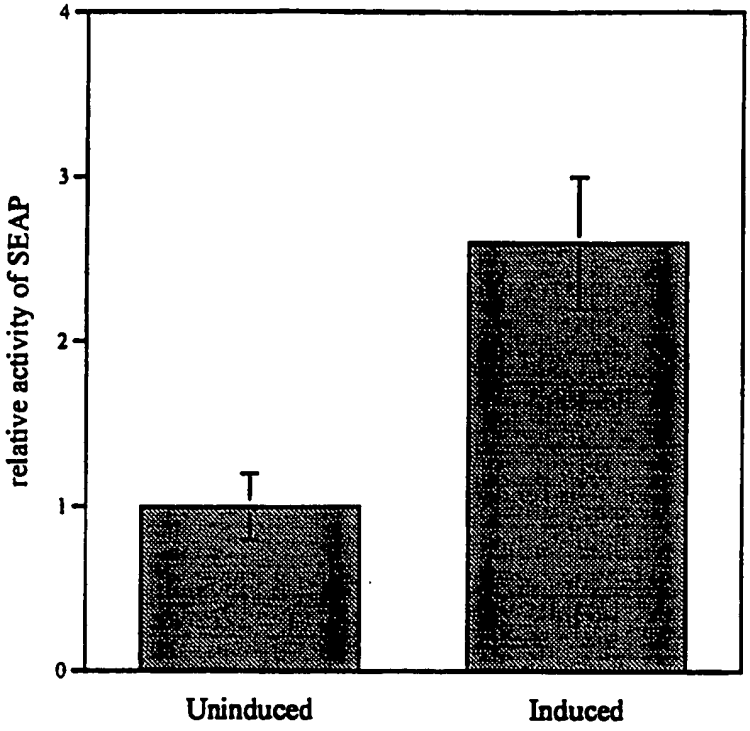


Figure 13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/13870

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.

US CL :435/69.1, 172,1, 172.3, 358, 410

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 172,1, 172.3, 358, 410

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SAVITZ et al. Identification of a ribosome receptor in the rough endoplasmic reticulum. Nature, 09 August 1990, Vol. 346, pages 540-544, see entire document.	1-17
A	WANKER et al. Functional Characterization of the 180-kD Ribosome Receptor In Vivo. The Journal of Cell Biology, July 1995, Vol. 130, No. 1, pages 29-39, see entire document.	1-17
Y	KALIES et al. Binding of Ribosomes to the Rough Endoplasmic Reticulum Mediated by the Sec61p-Complex. The Journal of Cell Biology, August 1994, Vol. 126, No. 4, pages 925-934, see entire document.	1-17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
09 SEPTEMBER 1998	13 OCT 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer IREM YUCEL Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/13870

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SAVITZ et al. 180-kD Ribosome Receptor is Essential for Both Ribosome Binding and Protein Translocation. The Journal of Cell Biology, February 1993, Vol. 120, No. 4, pages 853-863, see entire document.	1-17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/13870

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12P 21/06; C12N 5/00, 5/02, 5/06, 5/10, 15/00

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN (CAPLUS), DIALOG (MEDLINE)
Terms: rrp, ribosom? recept? protein?, polypeptide?, secret?, pathway?, machinery, membrane prolifer?, induc? increas?, enhanc? capacit?, transloc?, transport? inventors' names